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The Peroxisome Proliferator-Activated Receptor δ Agonist, GW501516, Inhibits Angiogenesis through Dephosphorylation of Endothelial Nitric Oxide Synthase

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Objective: Peroxisome proliferator-activated receptor δ (PPAR-δ) is an ubiquitously expressed nuclear receptor that has been implicated in adipose tissue formation, brain development, and atherosclerosis. Despite mouse studies demonstrating that PPAR-δ activation has favorable anti-atherogenic properties by improving systemic lipid profiles, the relationship between PPAR-δ agonist and angiogenesis is unknown. We hypothesized that PPAR-δ ligands modulate the angiogenesis.

Methods: To test this hypothesis we treated primary cultures of bovine aortic endothelial cells with PPAR-δ specific ligand, GW501516 (50-800 nM) for 6 h.

Results: GW501516 dose-dependently decreased nitric oxide production without alteration in endothelial nitric oxide synthase (eNOS) expression. Analysis with phospho-specific antibodies against eNOS demonstrated that GW501516 significantly decreased the phosphorylation of eNOS at Serine1179 (eNOS-Ser^{1179}). Concurrently, GW501516 also decreased the Akt phosphorylation. GW501516 did not affect endothelial cell proliferation or induce apoptosis. However, GW501516 inhibited endothelial cell migration, and tube formation in a high nanomolar concentration. The inhibition of endothelial cell tube formation by GW501516 was prevented by addition of the nitric oxide donor, DETA NONOate (5 μM). GW501516 was also found to inhibit angiogenesis *in vivo* in the chicken chorioallantoic membrane assay.

Conclusion: These results provide that high nanomolar range of GW501516 inhibits angiogenesis by a mechanism involving dephosphorylation of eNOS-Ser^{1179}.

Key Words: Aortic endothelial cells, GW501516, Nitric oxide, Angiogenesis

INTRODUCTION

Angiogenesis is a dynamic process of endothelial proliferation and differentiation. The formation of a functioning vasculature requires the orchestrated interaction of endothelial cells, extracellular matrix, and surrounding cells. A number of specific factors are known to stimulate or inhibit angiogenesis, including vascular endothelial growth factors (VEGF), inflammatory cytokines, adhesion molecules, and nitric oxide (NO).[1] Among these factors, NO is a critical mediator of angiogenesis. By enhancing endothelial cell survival, proliferation, and migration, NO is pro-angiogenic. VEGF, and other growth factors stimulate the endothelial elaboration of NO, which is a major mediator of their effects.[2] Angiogenesis plays a key role in many pathological conditions. For example, excessive angiogenesis is thought to promote and maintain tumor growth and metastasis, rheumatoid

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arthritis, retinopathy of prematurity, diabetic retinopathy, and atherosclerosis. Conversely, insufficient angiogenesis may be involved in diabetic patients having impaired collateral vessel development after coronary artery occlusion.[3,4]

Peroxisome proliferator-activated receptors (PPARs) are a subfamily of the nuclear receptor family of transcription factors that control the expression of key genes involved in the regulation of metabolism, inflammation, and thrombosis. Of the three PPAR isoforms (α, γ, and δ), PPAR-α activation by synthetic ligands (e.g., fenofibrates) has favorable anti-angiogenic properties.[5] PPAR-γ ligands, thiazolidinediones have been shown to enhance eNOS activity by phosphorylation in endothelial cells and to inhibit leukocyte-endothelial cell interaction. Moreover, 15d-PGJ_2, a PPAR-γ ligand, was reported to be a potent inhibitor of angiogenesis *in vitro* and *in vivo*.[6] PPAR-δ is an ubiquitously expressed nuclear receptor that has been implicated in adipose tissue formation, brain development, placental function, wound healing, and atherosclerosis.[7-11] However, the corresponding effects of PPAR-δ agonists on eNOS activity and the angiogenesis have not been explored.

In the present study, we investigated to determine the effects of PPAR-δ activation in NO production and angiogenesis in endothelial cells.

MATERIALS AND METHODS

1. Reagents

GW501516, a specific PPAR-δ agonist, was obtained from Calbiochem (La Jolla, CA, USA). Antibody against eNOS was purchased from Transduction Laboratories (Lexington, KY, USA). Antibody against p-eNOS-Ser^{1179} was obtained from Cell Signaling Technology (Beverly, MA, USA). Dulbecco's minimal essential medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), fetal bovine serum (FBS), antibiotic-antimycotic, L-glutamine, trypsin-EDTA solution, and plasticware for cell culture were purchased from Gibco-BRL (Gaithersberg, MD, USA). Unless otherwise indicated, all other reagents were purchased from Sigma (St. Louis, MO, USA)

2. Cell culture

Primary cultures of bovine aortic endothelial cells (BAECs) were done from isolated bovine aorta as described by Kim et al. with minor modifications.[12] In brief, the aortae were obtained from freshly slaughtered cattle and washed in DPBS and the luminal side of the aorta was subjected to 0.05% collagenase digestion for 10 min. BAECs were obtained by repeated gentle pipetting on the luminal side and were washed twice by centrifugation at 100 × g for 5 min at 4°C in DMEM containing 10% FBS, 1% antibiotic-antimycotic (penicillin G sodium, streptomycin sulfate and amphotericin B) and maintained in DMEM supplemented with 10% FBS at 37°C under 5% CO_2 in air. The majority of cells exhibited typical cobblestone configuration. The BAECs in culture were identified by positive staining for factor VIII-related antigen. Cells between passages 5 and 9 were used for all experiments. When BAEC were grown to confluence, cells were further maintained for 6 h in DMEM supplemented with 2% FBS, and after that the cells were treated or not treated with various concentrations of GW501516 for the indicated time.

3. Measurement of NO from BAECs

NO production by BAECs was measured as nitrite (a stable metabolite of NO) concentration in cell-culture supernatants, as described previously with minor modifications.[13,14] Briefly, at the end of the experiments the culture medium was changed to Kreb's solution (pH 7.4; 1 ml/40 mm dish) containing (in mM) NaCl 118, KCl 4.6, $NaHCO_3$ 27.2, $MgSO_4$1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, and glucose 5.5, and was equilibrated for 1 h at 37°C. At the end of the incubation, 200 μL of each supernatant

(in Kreb's solution) was carefully transferred into a 96-well plate, with the subsequent addition of 80 μL of Griess reagent (40 μL of 1% sulfanilamide containing 5% phosphoric acid and 40 μL of 0.1% N-(1-naphthyl) ethylenediamine). After color development at 25°C for 10 min, the absorbance was measured on a microplate reader at a wavelength of 548 nm. Each sample was assayed in duplicate wells. A calibration curve was plotted using known amounts of sodium nitrate solution. With this protocol, the measured values represent the amounts of NO produced by the cells during the 1 h incubation in Kreb's solution, following 6 h various concentrations of GW501516 treatment. Therefore, subsequent NO production was solely dependent on eNOS activity at the end of these treatments.

4. Western blot analysis

For western blot analysis, cells were washed with ice-cold DPBS and lysed in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 mM β-glycerophosphate, 1 mM NaF, 1 mM Na_3VO_4) containing 1 × Protease Inhibitor Cocktail™ (Roche Molecular Biochemicals, Indianapolis, IN, USA). Protein concentrations were determined with the BCA protein assay kit. Equal quantities of protein (30 μg) were separated on sodium dodecyl sulfate-polyacrylamide gel under reducing conditions, then electrophoretically transferred onto nitrocellulose membranes. The blots were then probed with the appropriate antibody directed against eNOS (1:4000), p-eNOS-Ser^{1179} (1:1000), Akt (1:4000), or p-Akt-Ser^{473} (1:1000), followed by the corresponding secondary antibody, and finally developed using enhanced chemiluminescence reagents (ECL, Amersham, UK).

5. Cell viability assay

The cell viability was assessed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay as reported previously.[15] Briefly, BAECs were seeded at density of 8×10^3 cells per well into 96-well culture plates. After attachment, the medium was changed to DMEM/0.5% FBS and incubated in the presence of DMSO or GW501516 for 24 h.

6. Cell migration assay

BAECs migration was assessed using a wound migration assay as described previously.[16] After wounding the compactly grown BAECs with a razor blade making the injury line, wounded BAECs were incubated in DMEM with 2% FBS, 1 mM thymidine (for cell proliferation inhibition), and 800 nM GW501516. BAECs were allowed to migrate for 16 h, fixed with absolute methanol, and stained with Giemsa's staining solution. Migration was quantified by counting the number of cells that moved beyond the injury line. This experiment was conducted independently three times.

7. Tube formation assay

To assess the inhibitory effects on capillary-like tube formation of BAECs by GW501516, changes in cell morphology were observed using a modification of the method described previously.[17] Briefly, Matrigel-coated 48-well plates were incubated for 1 h at 37°C to form a gel. BAECs diluted in 0.5% DMEM in the presence and absence of 800 nM GW501516 were added to each well at a density of 5×10^4 cells/well. After 6 h of incubation, a picture of the cell morphology was taken with a microscope.

8. Chicken chorioallantoic membrane (CAM) assay

The CAM assay was used for determining anti-angiogenic activity by the method as described previously.[18,19] In brief, fertilized chicken eggs were incubated in a constant-humidity egg breeder at 37°C. After three days of incubation, about 2-3 mL of albumin was aspirated from the eggs. GW501516 were applied to the 4.5-day-old

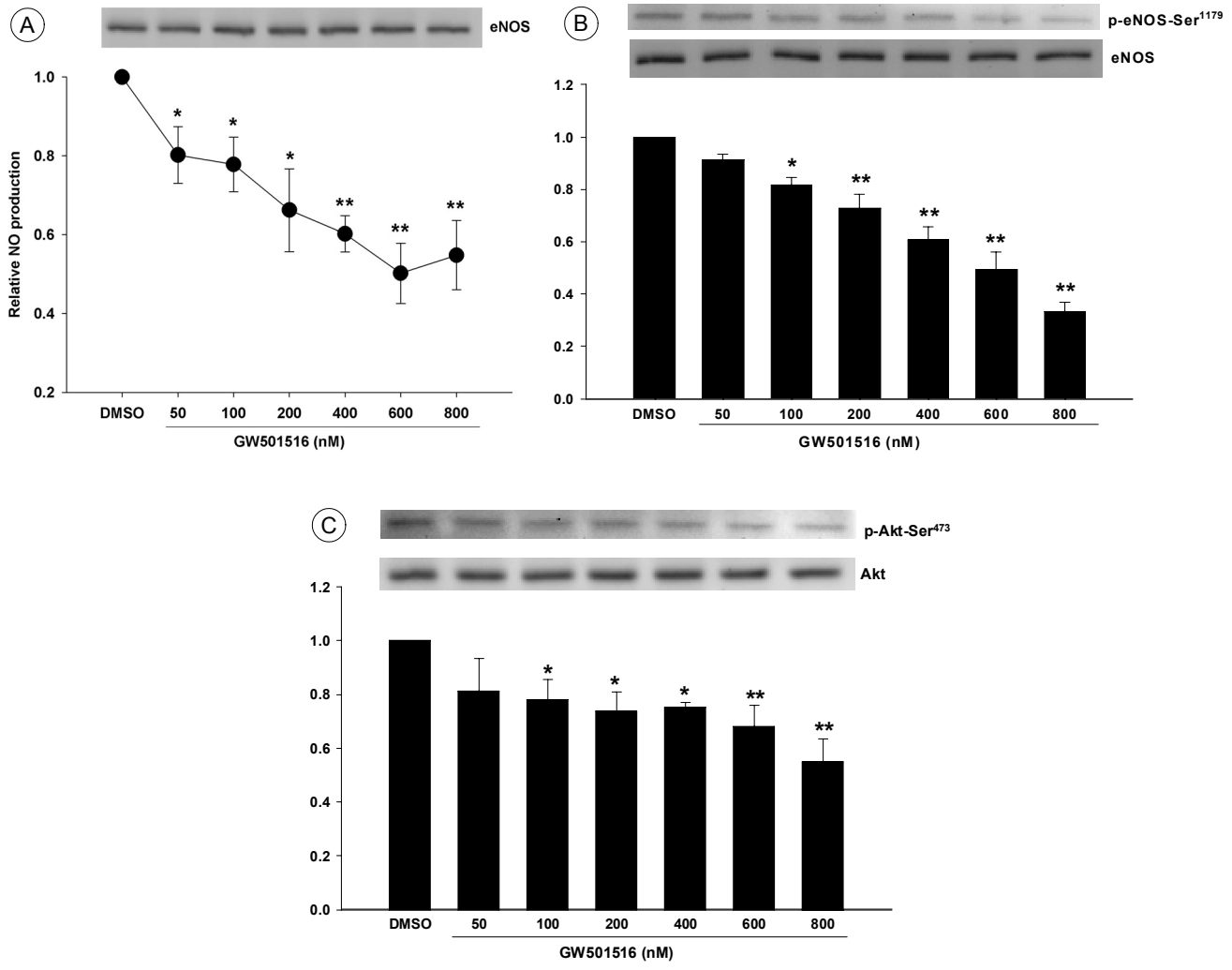

Fig. 1. Effect of GW501516 according to dose on NO production in BAECs. (A) Dose-dependent decrease of NO production in BAECs without alteration of eNOS. (B) GW501516 inhibited phosphorylation of eNOS-Ser^{1179} (C) GW501516 decreased phosphorylation of p-Akt-Ser^{473}. Data are means± SD of triplicate experiments. *P<0.05, **P<0.01 by ANOVA.

CAM at a dose of 2.5-10 μg/CAM. After 48 h incubation, 10% fat emulsion (Intralipose) was injected into the CAM and observed under a microscope.

9. Statistical analysis

All values are presented as the mean±SD. Statistical analyses were performed using either a Student's paired t-test or a one-way analysis of variance (ANOVA) using SPSS for Windows, version 11.0 (SPSS, Chicago, IL). A two-tailed P<0.05 was considered significant.

RESULTS

1. GW501516 decreased NO in BAECs without alteration of eNOS

As shown in Fig. 1A, GW501516 decreased NO production in a dose-dependent manner in BAECs (P< 0.05). Western blot analysis revealed that the decreased NO production did not result from a decrease in eNOS protein expression, suggesting that classical intracellular genomic activity is not responsible for this effect.

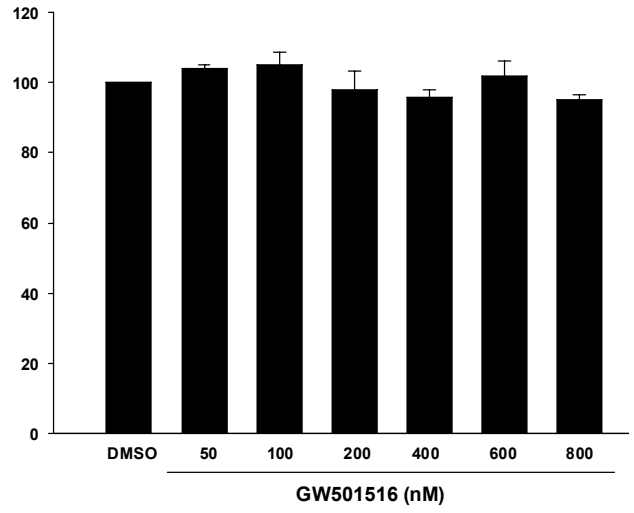

Fig. 2. Effect of GW501516 on cell viability in BAECs. Data are means±SD of triplicate experiments.

2. GW501516 decreased NO in BAECs by increasing eNOS–Ser^{1179} dephosphorlation

We examined extensively characterized phosphorylation site of eNOS on serine residues.[20] As shown in Fig. 1B, GW501516 increased eNOS-Ser^{1179} dephosphorylation in a dose-dependent manner. GW501516 also increased p-Akt-Ser^{473} dephosphorylation, which is down-stream signal of eNOS phosphorylation (Fig. 1C).

3. GW501516 did not affect BAECs proliferation or induce cell death

Cell viability was assessed using the MTT assay. BAECs treated with various concentrations of GW501516 showed viability of 90-110%, which was not statistically different (P>0.05) as compared to the viability of untreated control cells (Fig. 2). Therefore, GW501516 enhanced NO production without inducing cell death in BAECs.

4. Inhibition of migration of endothelial cells by GW501516 in BAECs

The effect of GW501516 on migration of endothelial cells was evaluated using the wound migration assay. As shown in Fig. 3, GW501516 at 800 nM showed a significant difference in the migration of cells when

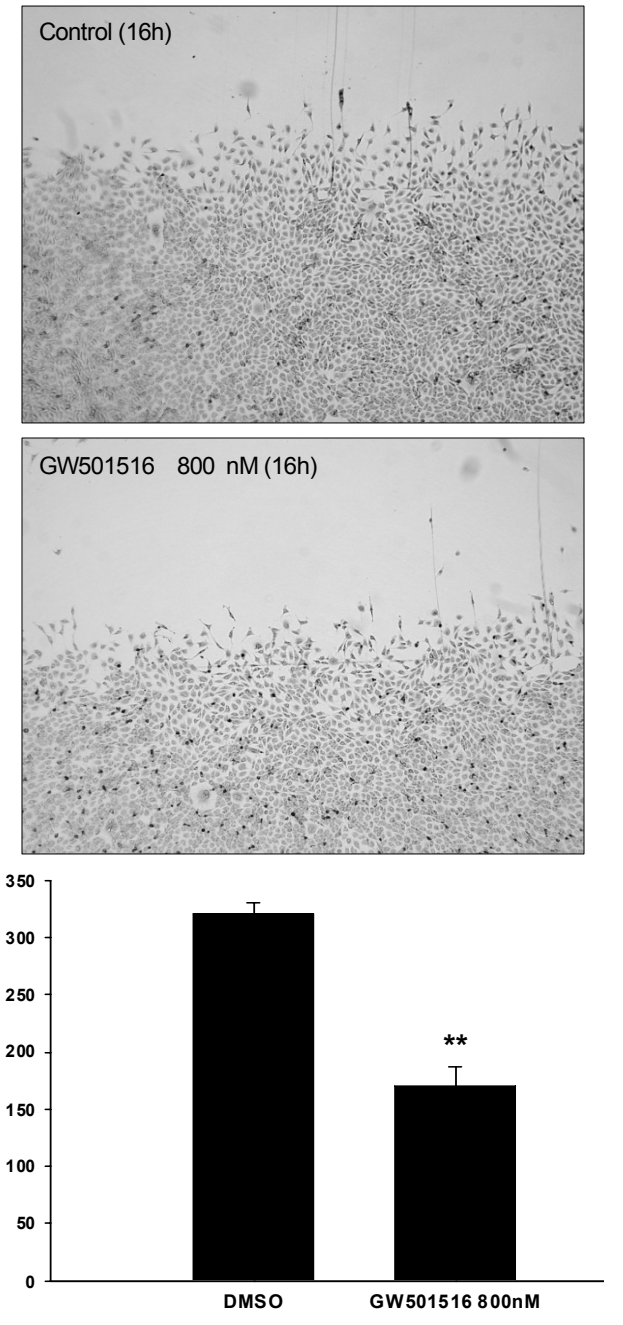

Fig. 3. Effect of GW501516 compared with DMSO on the migration of BAECs. BAECs wounded with a razor were treated with/without 800 nM GW501516 for 16 h and the migrated cells were counted. Migration was quantified by counting the number of cells that moved beyond the injury line. Data are means±SD of triplicate experiments. **P<0.01 by Student's paired t-test.

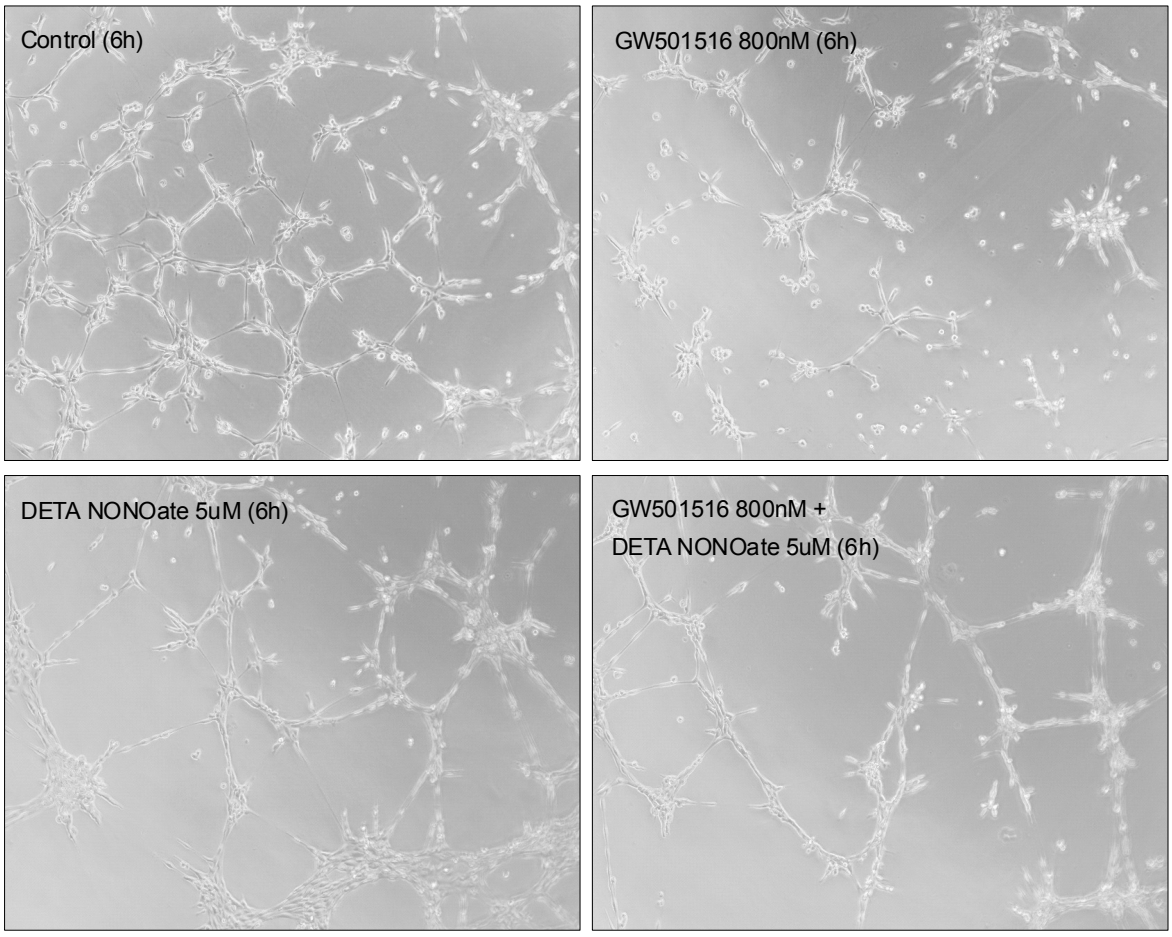

Fig. 4. Influence of GW501516 treatment on tube formation of BAECs. The effect of GW501516 on the morphological changes of BAECs on the surface of Matrigel was investigated. BAECs were grown on 48-well plates pre-coated with Matrigel and GW501516 for 6 h after seeding. The endothelial morphological changes were captured through an inverted microscope (40×) and photographed. Data are representative results from three separate experiments.

compared with a control ($P<0.01$).

5. Inhibition of tube formation by GW501516 in BAECs

We conducted *in vitro* experiments using Matrigel in BAECs. When plated on Matrigel, BAECs formed hollow tubes with lumen on Matrigel beds. These tubes became stronger and more robust with longer networks as time went on. In contrast, the addition of 800 nM GW501516 in Matrigel caused an inhibition of Matrigel-induced network formation of BAECs, resulting in less extensive, broken, foreshortened, and much thinner vessels at many sites when compared with the control. However, cells co-treated with a combination of GW501516 and the NO donor DETA NONOate (5 μM) demonstrated a normal angiogenic response comparable with that of cells treated with the NO donor alone (Fig. 4).

6. Anti-angiogenic effects on the treated chicken CAM due to GW501516

Anti-angiogenic activities of GW501516 were investigated using CAM assay. A marked inhibition of angiogenesis was seen on examination 2 days after GW501516 (2.5, 5, or 10 μg)-loaded thermanox coverslips

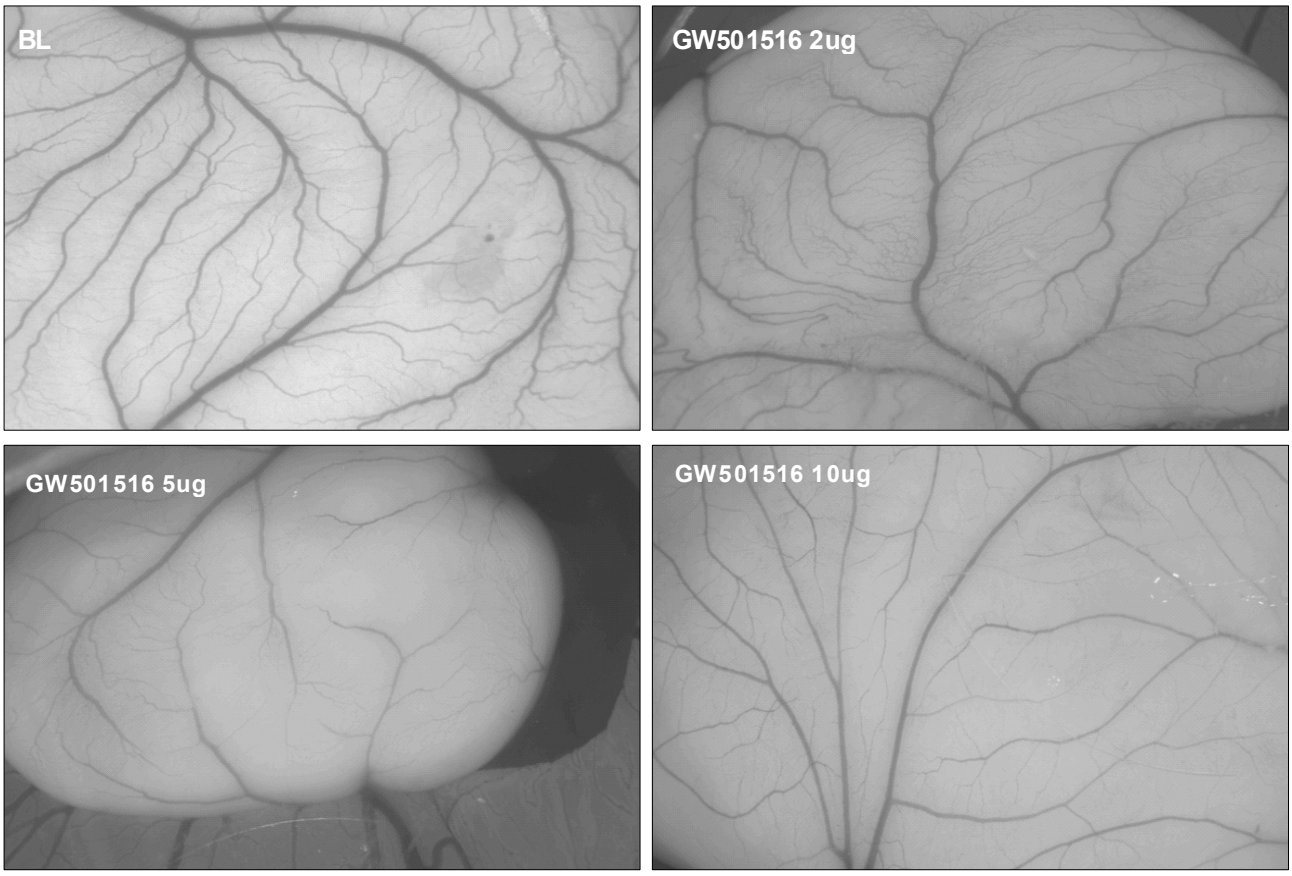

Fig. 5. In situ inhibition of angiogenesis in the chicken CAM. Fat emulsion (10%) was injected into the CAM to make the vascular network clear. Data are representative results from three separate experiments.

were placed at the vascular membrane when compared to DMSO-treated controls (Fig. 5).

DISCUSSION

PPAR-δ is expressed in a variety of cell types and plays important roles in mediating the action of development and physiology of various tissues such as adipose tissue, placenta, skin and small intestine.[21-24] Beside these actions on development, recent studies, revealed that targeted activation of PPAR-δ based on the utilization of PPAR-δ synthetic agonist, GW501516 in either adipocytes or muscles showed that it selectively activates genes of fatty acid oxidation and energy uncoupling and thus results in a lean phenotype.[25] In addition, long-term treatment of GW501516 causes dramatic weight loss accompanied with improvement in lipid profile.[26] These observations clearly indicated that activation of PPAR-δ has potential therapeutical interest in the obese state. Some PPAR-α or -γ ligands have been described as not only important regulators of adipogenic differentiation and energy metabolism, but angiogenic modulators.[5,6] These findings prompted us to study the anti-angiogenic potential of GW501516.

GW501516 (less than 1 μM) was used in all experiments, as used in cell culture experiments by Oliver et al.[26] to induce a reproducible maximal PPAR-δ response. Moreover, at this concentration Oliver et al. demonstrated that GW501516 was highly selective and did not activate or bind PPAR-α or -γ and other nuclear receptors. Our

results showed that the anti-angiogenic effects of GW501516 were observed *in vitro* at concentrations at 800 nM. Even though GW501516 activates human PPAR-δ with an EC_{50} of 1 nM and the effects at high nanomolar concentration of GW501516 might be nonspecific to human cells. However, the high nanomolar range of GW501516 used in this study might be possible to the BAECs because GW501516 does not activate PPAR-α or -γ at concentration of less than 1 μM.

eNOS is one of three NOS isoforms that catalyze the formation of NO and L-citrulline by the oxidation of L-arginine. The cardiovascular importance of this reaction relies on the formation of NO, a signaling molecule that regulates endothelial cell growth, survival, and angiogenesis.[27,28] eNOS knockout mice have been shown to exhibit marked impairment in angiogenesis.[29,30] BAECs are known to express eNOS, but not inducible NOS.[13] Therefore, the NO production in BAECs is solely dependent of eNOS. eNOS is not only controlled chronically by inducing its expression levels (e.g. shear stress) but acutely by regulating its enzyme activity involving eNOS-interacting proteins, posttranslational regulation, cofactors and substrates, subcellular localization.[31-33] Among these regulatory mechanisms, eNOS phosphorylation has been recognized as a critical mechanism. There are at least five specific phosphorylation sites and eNOS-Ser^{1179} is particularly well studied.[34,35] Our results showed that GW501516 attenuated eNOS activity through dephosphorylation of eNOS serine residue.

Because GW501516 decreased NO production and supplementation of GW501516-treated cells with an exogenous NO donor prevented the GW501516-induced inhibition of tube formation, the antiangiogenic effect of this PPAR-δ agonist seems to be linked to interference with NO-mediated signaling. Although the mechanism underlying this effect remains to be determined, the results obtained underscore the importance of NO signaling in the regulation of angiogenesis. However, we failed to detect any effects of exogenous NO donor on GW501516-treated BAECs' migration. The CAM assay is an important in vivo model of microvessel formation.[36] Antiangiogenic effects of GW501516 were also evident in vivo CAM assay based on reduced vessel ingrowth, the development of irregular and brittle vessels, and a markedly reduced perfusion compared with controls.

In conclusion, we report here that GW501516, a specific PPAR-δ agonist, decreases NO production and eNOS phosphorylation. Moreover, PPAR-δ agonist, in addition to improving lipid profile, might also provide anti-angiogenic effect and has a therapeutic value. It would be worthwhile verifying further the involvement of PPAR-δ in GW501516-mediated anti-angiogenesis by testing other applicable angiogenesis model (e.g. oxygen-induced retinopathy in mice) for its ability to inhibit angiogenesis.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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